2017

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Behavioural Brain Research 324 (2017) 115–124

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Cognitive performance of male and female C57BL/6J mice after repetitive concussive brain injuries

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HIGHLIGHTS

- Cognitive performance was assessed using pre-clinical assessments of learning and memory following repetitive concussive brain injuries.
- Female mice were observed to have reduced cognitive impairment and reactive astrogliosis following repetitive concussive injury.
- The discovery of sex differences following brain injury can provide the framework for future TBI remedies.

ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 24 January 2017
Accepted 11 February 2017
Available online 15 February 2017

Keywords:
Sex differences
Traumatic brain injury
Learning and memory
Cognition
Behavior

ABSTRACT

In contact sports, repetitive concussive brain injury (rCBI) is the prevalent form of head injury seen in athletes. The need for effective treatment is urgent as rCBI has been associated with a host of cognitive, behavioral and neurological complaints. There has been a growing trend in the use of female animals in pre-clinical research, but few studies have investigated possible sex differences following rCBI. The goal of the current study was to determine any differences between male and female C57BL/6J mice on assessments of learning and memory after repetitive concussive injury. Following rCBI by impact to the scalp, male mice exhibited longer righting reflexes during acute recovery. In both sexes, there were no evident histopathological changes observed in the underlying cerebral cortex or hippocampus. Reactive astrogliosis was elevated in the corpus callosum and optic tract, and astrogliosis was slightly less in the optic tract of female mice. rCBI mice exhibited impairment during the learning phase of the Morris water maze (MWM), but female mice, in comparison to male mice, were observed to have superior spatial memory during standard MWM probe trials. Female mice were overall more active, evidenced by greater distances traveled in the y-maze and greater swim speeds in the MWM. The results of this study demonstrate sex differences in cognitive performance following rCBI and support previous research suggesting the neuroprotective role of sex in brain injury.

1. Introduction

Traumatic brain injury (TBI) has become a primary concern in the world of sports. While reports of TBI in professional athletes tend to receive a majority of the media's attention, the incidence of TBI is greater among youth and non-professional athletes, where over 170,000 non-professional athletes are treated annually for sports-related brain injuries [1]. However, this value likely underrepresents the true number of athletes affected by TBI. Reports of TBI incidence do not account for those athletes who avoided seeking medical attention for their injury. Furthermore, due to the difficulty of detecting TBI with routine neuroimaging techniques, an unknown proportion of athletes with milder forms of TBI will be undiagnosed and, therefore, unreported.

In contact sports such as soccer, hockey, and American football, repetitive concussive brain injury (rCBI) results from the high velocity impacts that cause rapid acceleration and displacement of the head. Compared to athletes with a single CBI, athletes with rCBI have a greater susceptibility to subsequent concussive injuries as well as prolonged symptom duration [2,3]. Repetitive CBI

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http://dx.doi.org/10.1016/j.bbr.2017.02.017

has been associated with a variety of neuropathological changes, including neurochemical imbalances, impairment of blood-brain barrier (BBB) integrity, persistent, low-grade neuroinflammation, increased astrocytic and microglial response, diffuse axonal injury, and brain volume loss [4–6], and recent studies have suggested a link between repeated head trauma and the development of chronic traumatic encephalopathy (CTE), a progressive neurodegenerative disease [7–9].

Currently, there are no fully effective therapies that target brain injury. In order to develop effective treatments, further research is needed to better understand the pathophysiological mechanisms of rCBI. The use of animal models provides researchers with a method for studying the neuropathological and behavioral effects of brain injury, where animal models of rCBI have been suggested to closely resemble rCBI in humans [10]. Unlike traditional open-head injury models, such as controlled cortical impact (CCI) and fluid percussion (FP), rCBI models produce mild, closed-head injuries. By using repetitive, closed-head models, researchers have been able to eliminate the more severe injury effects from CCI and FP, while still imparting the sequelae of rCBI. Kane and colleagues [10], for example, developed a modified version of the Marmarmou [11] weight drop method to deliver multiple impacts over one or two weeks. While rCBI-mice exhibited deficits in motor coordination and mild astrocytic activity, severe outcomes such as cranial fracture, edema, seizures, and BBB breakdown were avoided [10].

Assessments of learning and memory are widely used in pre-clinical research to determine the duration and severity of cognitive outcomes following rCBI. Using tests such as the Morris water maze and the Barnes maze, researchers have found significant impairments in visuospatial learning and short-term memory after multiple concussions [12–16]. In comparison to single-CBI animals, these cognitive deficits have been observed to persist significantly longer in animals with rCBI, lasting anywhere from three months to over a year [12,13,15,16]. Furthermore, studies utilizing reversal paradigms for the Morris water maze find animals with rCBI to have difficulties relearning and retaining information [14].

Overall, animal models of rCBI have allowed researchers to better replicate the human form of concussion while cognitive assessments continue to aid in the evaluation of neurobehavioral functioning post-injury. However, the majority of TBI studies lack the inclusion of female animals, making it difficult to translate findings to the whole human population. The lack of female data is a cause for concern as the rate of TBI in female athletes has steadily increased over the last decade [17]. To address the problem of male over-reliance, the National Institutes of Health (NIH) have required the use of female animals or cells in all pre-clinical research [18]. The inclusion of female animals in pre-clinical TBI research is critical. Further investigation is needed to determine the neuropathological and behavioral differences, if any, between males and females following TBI, since knowledge of any sex differences could influence how medical practitioners treat TBI. Therefore, the aim of this work was to observe the performance of male and female C57BL/6J mice on cognitive assessments of learning and memory. To our knowledge, this is the first study to examine sex differences in functional outcomes following rCBI.

2. Methods

2.1. Animals and CBI procedures

Male and female C57BL/6J mice approximately 9 weeks old were obtained from Jackson Laboratories (Cat. No. 0664, Bar Harbor, ME), group-housed (3–5 mice per cage) and allowed to acclimate to housing facilities for approximately one week prior to TBI procedures. Facilities are approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were provided with food (Harlan Teklad Global Diets 2018, 18% protein) and filtered tap water *ad libitum* and were on a standard 12-h light-dark cycle; all testing was performed during the light phase of the cycle and behavioral testing was shared by male and female investigators [19]. All procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences. Male and female mice weighed 22.8 g–30.3 g and 16.2 g–23.2 g on the first day of CBI or sham procedures, respectively. Female mice were at random (undetermined) stages of the estrus cycle. Cages of mice were randomly assigned to receive 5x CBI (male, $n = 19$; female, $n = 19$) or 5x sham (male, n = 18; female, n = 19) procedures. Multiple (5x) CBI and sham procedures were performed at 24 h intervals. Mice were anesthetized in a clear induction chamber with 3% isoflurane (Forane, Baxter Healthcare Corporation, Deerfield IL) until corneal and pedal reflexes were absent. Once anesthetized, head hair was clipped and Nair depilatory cream (Church & Dwight, Princeton, NJ) was applied to remove all fur. Mice were then placed into a stereotaxic device with an incisor bar and atraumatic ear bars; anesthesia (1.5% isoflurane) was maintained via a flow-through nose cone. Under bright illumination, the suture of the cranium was visualized underneath the skin and the skin location marked by a permanent marker with a small dot. Concussive impacts were performed with the Impact One™ device (Leica Microsystems, Buffalo Grove, IL). The 5.0-mm-diameter steel tip was centered over the injury site (2.5 mm posterior to bregma and 2.5 mm left of bregma) at a 15-degree angle relative to the sagittal plane; tip contact with skin was confirmed by auditory feedback from the device. Isoflurane was discontinued immediately prior to injury delivery (continuation of 100% oxygen). The impact was delivered with a velocity of 5.0 m/s, dwell time of 0.1 s and a depth of 1.2 mm. Apnea following injury was measured, and animals were placed into a warm cage in a supine position. The righting reflex (amount of time before the animals turned completely to a prone position) was measured. Sham-treated mice underwent all procedures except the impact. All mice received acetaminophen in their drinking water (1 mg/ml)

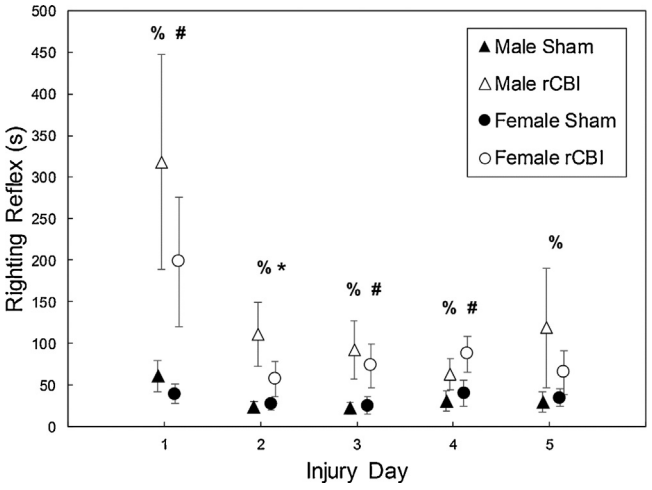

Fig. 1. Mean righting reflexes with 95% confidence intervals (based on z-scores) in male and female mice following concussive brain injury (CBI) on five consecutive days. Kruskal-Wallis tests were performed to compare the injury and sex effects on each day. Male mice that sustained CBI had significantly longer righting reflexes than male sham controls on all five days. Female mice with CBI had longer righting reflexes than female sham controls on days 1, 3 and 4 only. On day 2, the injured female mice righted themselves sooner than the male injured mice. The asterisk (*) represents a difference between the male and female injured groups on the given day; the pound sign (#) represents a difference between injured female mice and female sham controls, and the percent sign (%) indicates that CBI increased the righting reflex in male mice compared to sham controls.

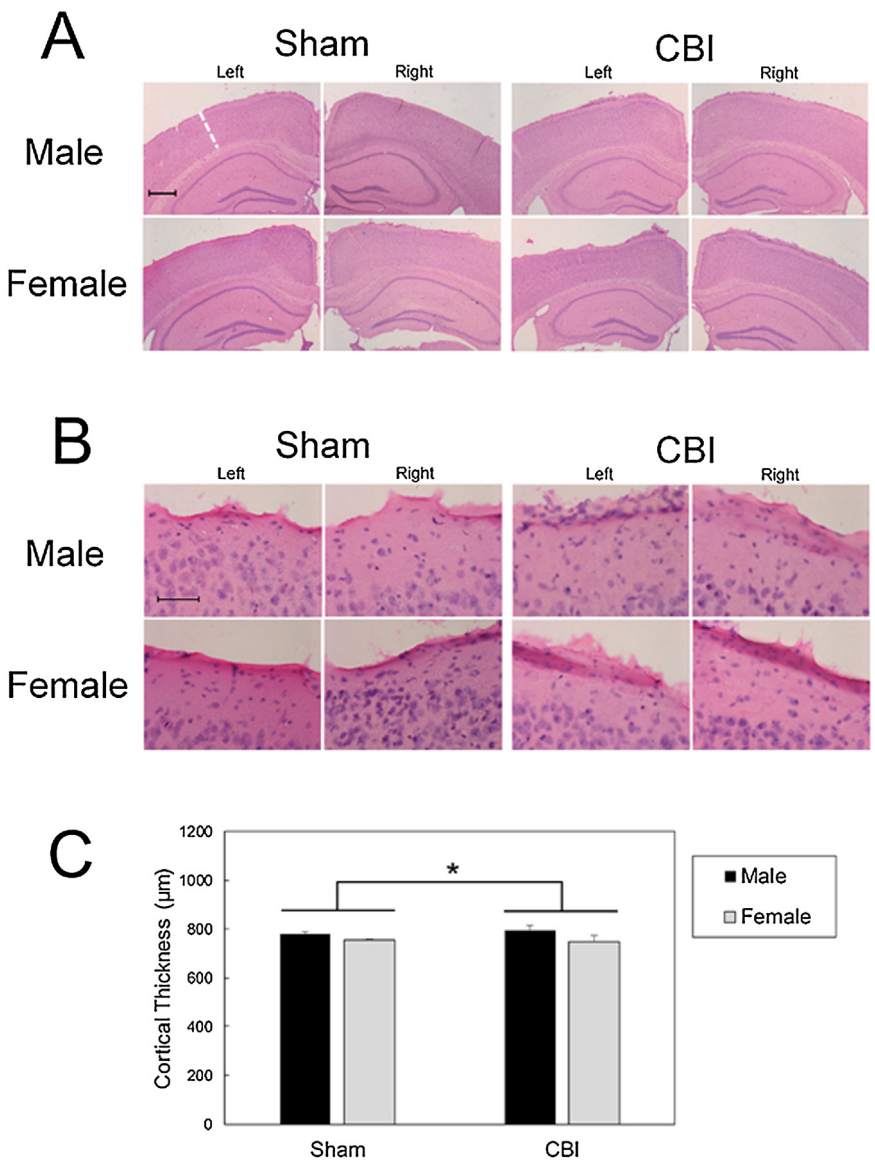

Fig. 2. H&E. Neuropathology 32 days following five concussive brain injuries (one day interval between injuries). Sections shown at 4x (A) or 40x (B) and the white dashed dashed line in the upper left figure of (A) shows the position and orientation used to measure cortical thickness. There was no difference between injured and sham control mice in cortical thickness (C), but there was a main effect of sex, with female mice having thinner cortex than male mice. Scale bar in (A) represents 500 μm, scale bar in (B) represents 50 μm. The asterisk (*) represents a main effect of sex. H&E, Hematoxylin and eosin; CBI, concussive brain injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from the time period immediately after the first injury until approximately 24 h after the final injury.

2.2. Cognitive assessments

The y-maze test of spontaneous alternation and Morris Water Maze (MWM) test were performed as previously described [20]. Briefly, on the 12th day following the final injury or sham procedure, mice were placed into the y-maze apparatus and allowed to freely explore for 5 min. Movements of the animals were recorded by an overhead camera coupled with a computer with Any-Maze software (Stoelting Co, Wood Dale, IL) that recorded movements of the animals and reported the total distance traveled for each mouse. Entries into the three arms were scored offline from videos by an observer blinded to the sex and injury conditions of the mice. A visit to an arm was counted when all four paws entered the arm. Visits to three different arms consecutively was counted as an alternation, and the percent correct alternation was calculated as $100 \times \frac{total\ number\ of\ alternations}{total\ arm\ entries - 2}$.

Standard MWM training trials were performed on days 17–20 following the final CBI or sham procedure. The MWM tank was 122 cm in diameter and filled with water at 23 ± 1 °C. A transparent platform was submerged about 1 cm below the surface of the water and located approximately 15 cm from the edge of the tank, and prominent visual cues were on the walls around the apparatus. Each training day, each mouse underwent four trials; the mouse was placed into the tank at a different location for each trial, facing the wall. The mouse was allowed 60 s to swim and find the platform (after which it remained on the platform for 15 s); if the platform was not located during that time the animal was gently guided to the platform and allowed to remain there for 15 s. Following each trial, the animals were dried and placed into a heated cage for approximately 3–4 min before the next trial. An overhead camera connected to a computer with Any-Maze software (Stoelting Co., Wood Dale, IL) recorded all movements of the animals, and the

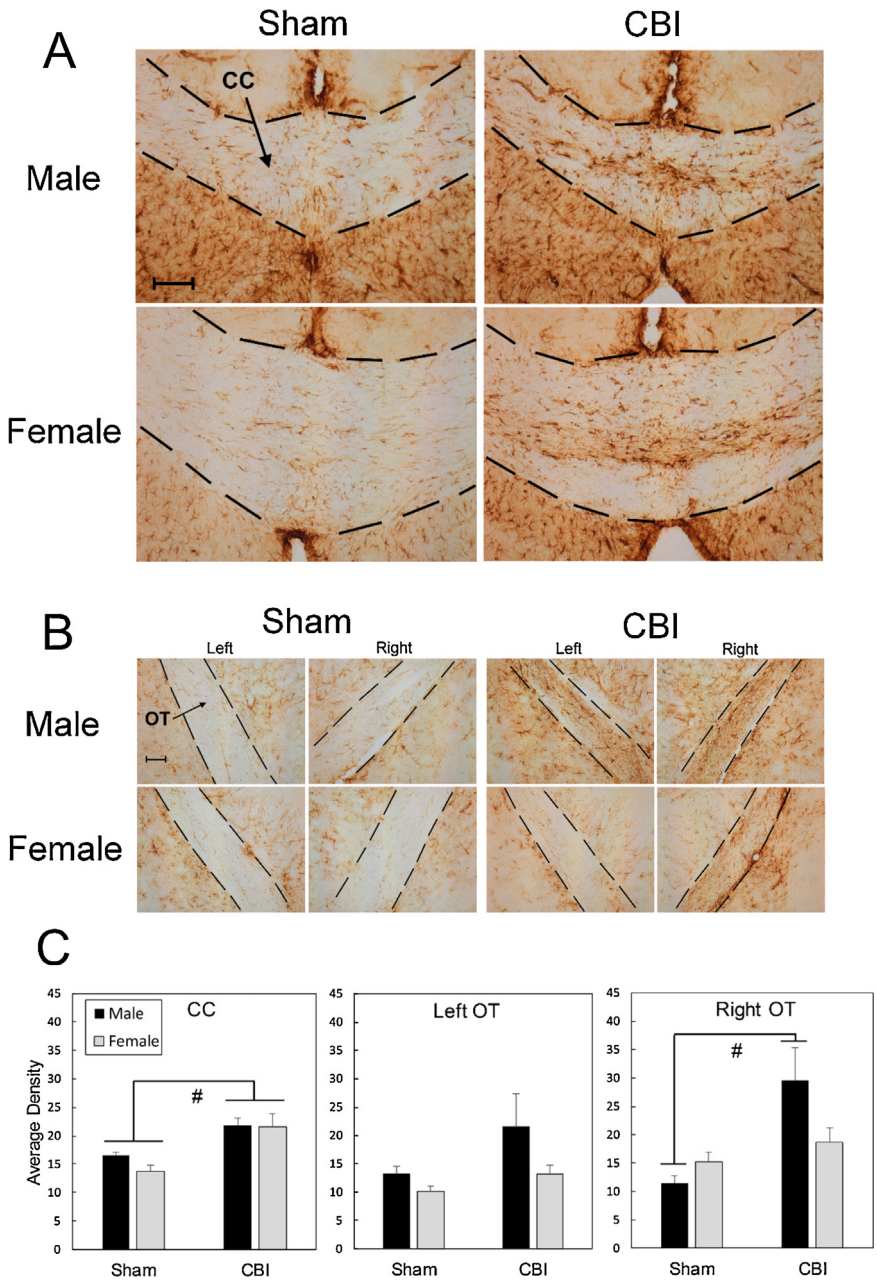

Fig. 3. GFAP. Increased astrocytosis in the corpus callosum (CC; A) and optic tracts (OT; B) in injured mice compared to sham controls. Quantification of GFAP staining density (C) showed that male and female injured mice had increased astrocytosis in the CC (C; left panel), but in the OT the only significant increase in staining density was in the right OT of injured male mice (C; right panel). Sections in (A) and (B) represent the approximate median of the animals that were processed for GFAP. Scale bar in (A & B) represents 100 μm. The pound sign (#) represents a difference between injured mice and sham controls. GFAP, glial fibrillary acidic protein; CC, corpus callosum; OT, optic tract; CBI, concussive brain injury.

software-reported measures included swim speed, distance swam before reaching the platform, and latency to find the platform. Any animal that did not find the platform during the trial was assigned the maximum score of 60 s for the latency. These measures (speed, distance, latency) were averaged across the four trials on a training day, resulting in one value for each animal for each training day.

A probe trial took place approximately 24 h following the final training trial (day 21 following the final CBI procedure). The platform was removed from the MWM and each mouse was placed in the tank opposite the former location of the platform, facing the wall, and allowed 60 s to swim around the tank. Any-Maze software reported the amount of time each mouse spent in the quadrant of the MWM (NW) that formerly housed the escape platform.

Three days after the probe trial, reversal training trials were performed for four days (days 24–27 following the final CBI). These trials were performed identically to the initial four days of training, except the platform was relocated to the opposite quadrant of the MWM (SE). A reversal probe trial was conducted 24 h following the final reversal training trial (day 28 following final CBI procedure).

Finally, three days following the reversal probe trial (day 31 following the final CBI procedure), visible platform trials were performed. The platform, with a large patterned flag visible to the mice and marking the exact location, was placed into the center of the MWM. Four trials identical to the standard and reversal training trials were conducted, and the latency to locate and rest on the platform was recorded by Any-Maze software.

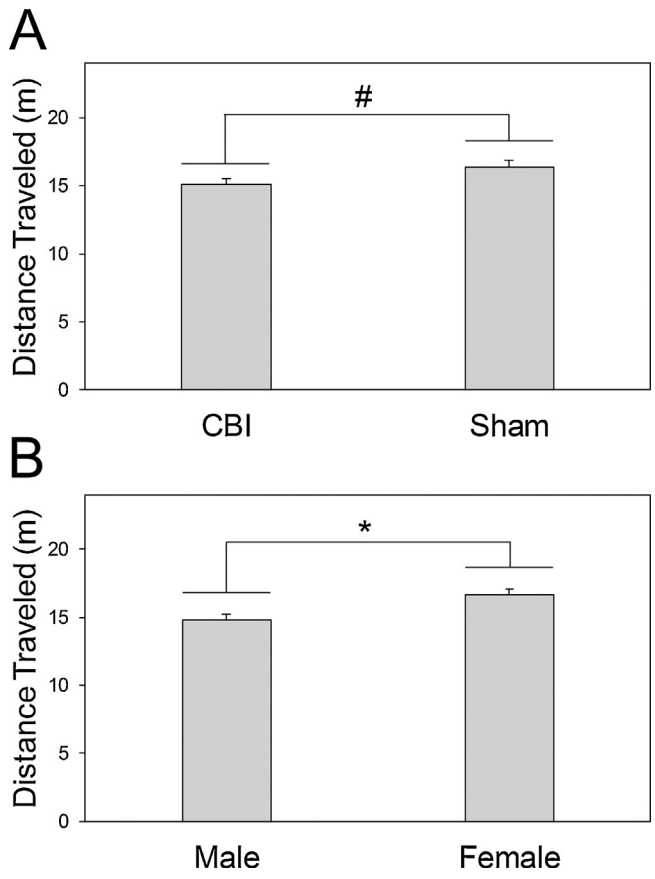

Fig. 4. Effects of sex and injury on ambulation in the y-maze. There was a main effect of injury on distance traveled in the y-maze (A; data collapsed by sex), with sham control ambulating greater distances in the apparatus than injured mice. Female mice were more active than male mice (main effect of sex; B; data collapsed by injury). The pound sign (#) represents a main effect of injury; the asterisk (*) represents a main effect of sex.

On day 32, all mice were deeply anesthetized and transcardially perfused with 0.1 M of phosphate buffer followed by 4% of paraformaldehyde (PFA) in 0.1 M of phosphate buffer. Brains were removed and post-fixed overnight in 4% PFA, followed by cryoprotection in 20% sucrose for 48 h. Brains were then frozen and sectioned (30 μm) with a sliding microtome, and sections were stored at −20 °C.

2.3. Histochemistry

Six animals from each sex and injury group were randomly chosen for immunohistochemical analysis. Coronal sections were stained using glial acidic fibrillary protein (GFAP) and hematoxylin and eosin (H&E) staining protocols.

2.3.1. Measurement of cortical depth

Sections were washed with PBS, mounted on slides, and allowed to air-dry overnight. The next day, slides were rinsed with distilled water three times for 1 min each followed by 2 min in 100% ethanol. Slides are incubated in hematoxylin (Sigma-Aldrich, GHS132) for 3 min and then rinsed with tap water for 5 min. Slides were quickly dipped into acid ethanol (37% HCl, 70% ethanol) 12 times and then rinsed with tap water for 3 min. Following an additional rinse in distilled water for 2 min, slides were incubated in eosin (Sigma-Aldrich, HT110332) for 30 s and then rinsed with tap water for 3 min. Slides were dehydrated through graded ethanol washes (95% and 100%), air-dried, and then cleared with xylene. Slides were coverslipped with Permount and allowed to dry overnight before viewing.

As a means of assessing changes in the cerebral cortex following CBI, H & E stained coronal sections from the sampled tissue was used to evaluate cortical depth. Using a Zeiss Axioskop with an attached AxioCam MR.5 camera and AxioVision software (version 4.7.2.0), samples of tissue at approximately bregma −1.70 mm in the coronal plane, according to the mouse brain atlas of Franklin and Paxinos, were used [21]. A line was drawn in the horizontal plane to approximately 1.75 mm lateral from the superior sagittal fissure. A vertical line was then located at this lateral location, the lateral parietal association cortex, and drawn in the vertical plane, but with an angle so that the line intersected with the tangential surface of the cortex. Cortical thickness was then measured from the surface of the cortex to the dorsal boundary of the corpus callosum (see white dashed line in upper left photomicrograph of Fig. 2A).

2.3.2. GFAP staining

Sections were washed with 1x TBS-Triton (0.05%) three times for 10 min followed by incubation in 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidases. The H_2O_2 solution was removed and sections were washed with TBS-Triton three times. Sections were blocked in blocking buffer (MOM™ Mouse IgG Blocking Reagent, Vector, MKB-2213) for 1 h at room temperature (RT). The primary antibody, GFAP (1:500; Thermo Fisher Scientific, Inc., MS-280-P), was diluted in blocking buffer, added to each section, and then incubated at 4 °C overnight. The next morning, the primary antibody solution was removed and sections were washed three times with 1x TBS-Triton. The biotinylated secondary antibody (1:500; AffiniPure Goat Anti-Mouse IgG [H + L], Jackson ImmunoResearch Laboratories, 115-065-003) was diluted in blocking buffer, added to each section, and then incubated for 1 h at RT. The secondary antibody solution was removed and sections were washed three times with 1x TBS-Triton. Sections were incubated in ABC reagent (Vector Labs, PK-4000) for 45 min at RT and then washed with 1 x TBS-Triton three times. DAB staining solution (Vector Labs, SK-4100) was added to each well and incubated for three min at RT, until sufficient color developed. DAB staining solution was removed and 1 ml PBS was added to each well to stop the DAB reaction. Sections were mounted on slides and allowed to air-dry overnight. The next day, sections were dehydrated through graded ethanol washes (75%, 85%, 95%, and 100%), cleared in xylene, and coverslipped with Permount (Fisher Scientific, SP15). Slides were allowed to dry overnight before viewing.

2.3.3. GFAP densitometry

Images of the corpus callosum (CC) and optic tracts (OT) were captured at 10 x magnification and densitometry was performed using a Zeiss Axioskop microscope with an attached AxioCam MR.5 camera and version 1.50i ImageJ software [22]. Areas of the CC and OT were traced via freehand selection and the measurement feature was employed to determine the mean grey density. The background was subtracted from each image by selection of an area with the absence of immunostaining. Average density values (i.e., density = mean grey density – background) for the CC and OT were calculated using three to four sections per animal. All assessments were made by a single investigator blinded to the status of sex and injury.

2.4. Statistical analyses

Statistical analyses were performed with SAS Studio 3.5 (SAS Institute Inc., Cary, NC). Data from MWM training trials were analyzed with a three-way mixed linear model (PROC MIXED) analysis of variance (ANOVA), with injury and sex as fixed factors and

training day as a repeated measure factor. Standard and reversal trial data were analyzed separately. An autoregressive (Lag-1) covariance structure and the Kenward-Roger degrees of freedom approximation were employed. There were no three-way interaction (injury x sex x day) effects found; only two-way interaction statistics are reported in the results section. Latency data from visible platform trials, as well as probe trial, y-maze, and immunohistochemical data were analyzed with two-way ANOVAs (PROC GLM), with sex and injury as fixed factors. Where significant sex by injury interactions were found, Bonferroni-corrected *t*-tests were performed on least square mean differences (PROC PLM). With the exception of GFAP density data from the left optic tract (OT), all data passed the homogeneity of variance test as assessed by Levene's test of the equality of variances. Inverse values of left OT data passed Levene's test and an ANOVA was performed on these values. Cohen's *d* (effect size) was calculated for significant results as $|\frac{\mu_{female}-\mu_{male}}{s_{pooled}}|$, where $s_{pooled} = \sqrt{\frac{s^2_{Female}+s^2_{Male}}{2}}$.

Righting reflex data did not pass the homogeneity of variance test as assessed by Levene's test of the equality of variances. SPSS (version 21; IBM SPSS Statistics, Armonk, NY) was employed to perform a Kruskal-Wallis test on the four sex/injury groups separately for each day, followed by stepwise step-down follow-up tests. Figures were created with Microsoft Excel 2013 and Daniel's XL Toolbox 6.60. Data in figures depicting behavioral data (Figs. 4–6) represent the means ± standard error of the mean; the righting reflex data set in Fig. 1 is represented by means with 95% confidence intervals.

3. Results

3.1. Extent of injury

During the immediate time following the injuries, apnea was rarely observed (data not shown), but the groups had significant differences in righting reflexes (Fig. 1) on all of the injury days (Day 1: $H(3) = 37.672$, $p < 0.001$, Day 2: $H(3) = 23.719$, $p < 0.001$, Day 3: $H(3) = 20.931$, $p < 0.001$, Day 4: $H(3) = 20.455$, $p < 0.001$, Day 5: $H(3) = 13.144$, $p = 0.004$). *Post-hoc* tests showed that the injured male mice had significantly longer righting reflex times than their sham counterparts on all injury days; injured female mice had longer righting reflex times than sham-treated females on days 1, 3 and 4, but these groups were equal on days 2 and 5. In addition, on day 2, the injured female mice had significantly shorter righting reflex times than the injured male mice.

As seen in Figs. 2A and B, H & E analysis indicated there was little gross observable damage at the injury site or in the hippocampus 32 days after final injury. Measurements of cortical thickness showed that there was no effect of repeated injury ($F_{1,20} = 0.107$, $p = 0.7566$), but there was a main effect of sex ($F_{1,20} = 4.555$, $p = 0.045$, Cohen's $d = 0.9045$), with female mice having a thinner measured cortex than male mice (Fig. 2C). Astrocyte reactivity as measured by density of GFAP staining was increased in the corpus callosum (Fig. 3A) of both male and female injured mice compared to sham controls (main effect of injury: $F_{1,19} = 20.940$, $p = 0.0002$; left panel, Fig. 3C), but there was no effect of sex ($F_{1,19} = 0.969$, $p = 0.3374$) or sex by injury interaction ($F_{1,19} = 0.726$, $p = 0.4047$). In the optic tract (OT) of mice that had sustained concussive brain injuries (Fig. 3B), there was no sex by injury interaction ($F_{1,18} = 0.250$, $p = 0.6321$) or main effect of either sex ($F_{1,18} = 2.689$, $p = 0.1184$) or injury ($F_{1,18} = 2.219$, $p = 0.1537$) on astrogliosis in the OT on the side (left) ipsilateral to the injury (center panel, Fig. 3C). On the side (right) contralateral to the injury, there was a significant sex by injury interaction ($F_{1,18} = 5.492$, $p = 0.0308$; right panel, Fig. 3C). Bonferroni-corrected *post-hoc* tests showed that male mice that had sustained repeated injury had significantly increased GFAP staining in the right OT compared to male sham controls ($p = 0.0051$, Cohen's $d = 1.9464$); levels of astrogliosis were equivalent in injured female mice and female sham controls ($p = 0.8493$).

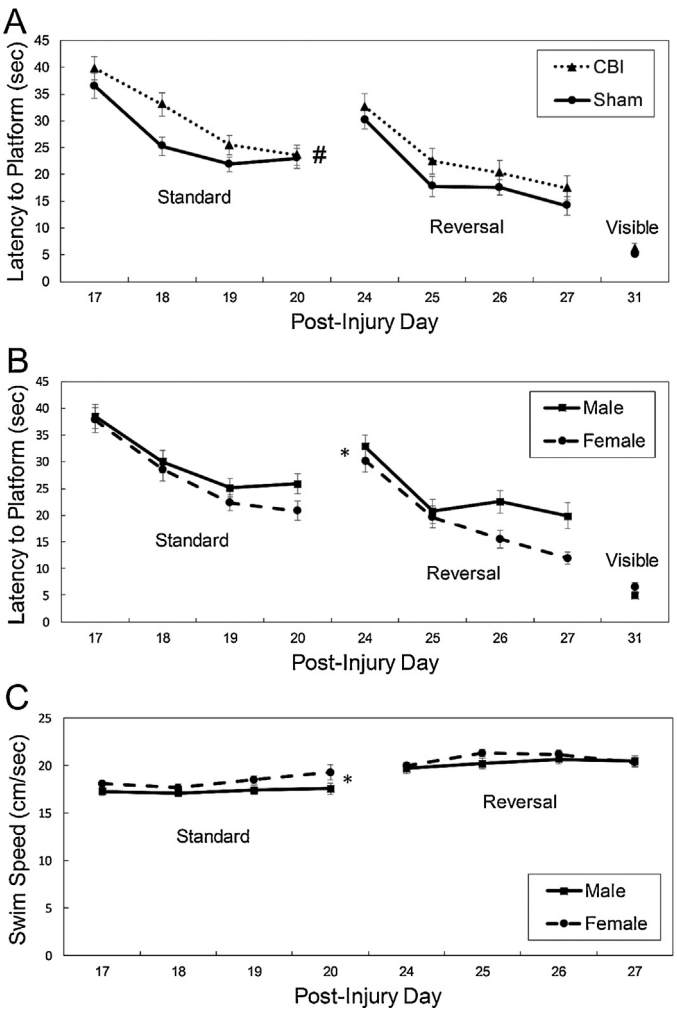

Fig. 5. Spatial training trials in the Morris water maze. There was a main effect of injury on the latency to find the platform during the standard training trials on days 17–20 following the final injury (A), with injured mice requiring longer times to locate the platform. Female mice outperformed male mice during the reversal training trials on days 24–27 following injury (B), locating the platform sooner. Female mice swam faster during standard, but not reversal, training trials (C). The asterisk (*) represents a main effect of sex; the pound sign (#) represent a main effect of injury.

3.2. Y-Maze

There was no sex by injury interaction effect ($F_{1,71} = 0.43$, $p = 0.5146$), main effect of sex ($F_{1,71} = 3.36$, $p = 0.0709$), or main effect of injury ($F_{1,71} = 0.11$, $p = 0.7378$) on spontaneous alternation behavior in the y-maze following CBI (data not shown). However, although there was no sex by injury interaction effect for ambulation in the y-maze as measured by the total distance traveled ($F_{1,71} = 3.74$, $p = 0.0572$), there were main effects of both injury ($F_{1,71} = 3.98$, $p = 0.0499$, Cohen's $d = 0.3915$) and sex ($F_{1,71} = 9.07$, $p = 0.0036$, Cohen's $d = 0.6711$) on activity levels in this test (Fig. 4A and B, respectively). Mice that had undergone sham procedures and female mice traveled greater distances than injured mice and male mice, respectively.

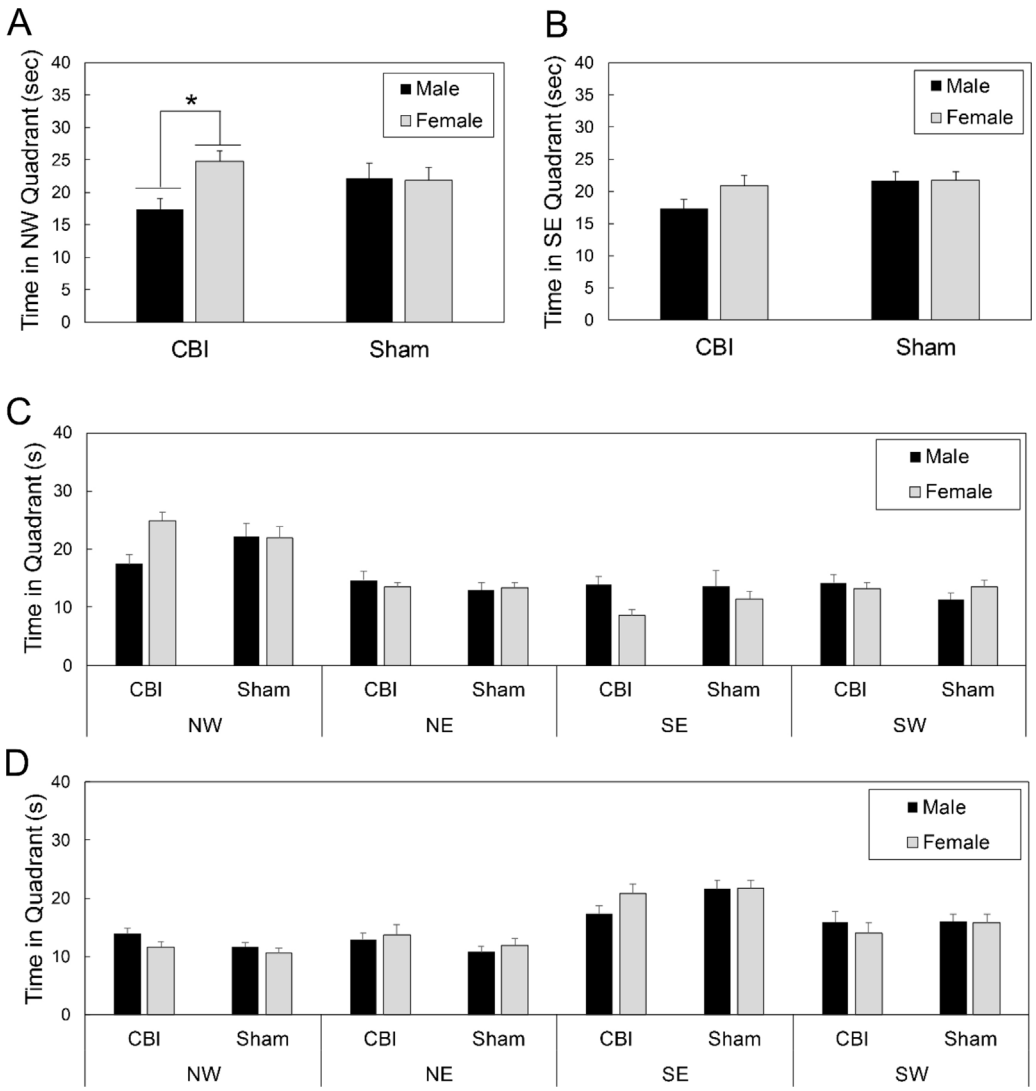

Fig. 6. Spatial memory in the Morris water maze as assessed by standard (A) and reversal (B) probe trials on days 21 and 28 following the final injury, respectively. There were no sex differences in performance in the sham controls during either the standard or reversal probe trial, but during the standard probe trial, the injured female mice outperformed the injured male mice, spending a greater amount of time in the target quadrant. The asterisk (*) represents a main effect of sex. Statistical analyses were only performed on the time spent in the NW quadrant (standard probe trial) or the SE quadrant (reversal probe trial), but the time spent in all platforms is shown in (C) for the standard probe trial and (D) for the reversal probe trial.

3.3. Morris water maze

In the MWM, no sex by injury interaction effects were found for any measures (speed, latency, distance) during standard or reversal training trials. The latency to find the platform was increased by brain injury during the standard training trials (injury by day interaction, $F_{3,162} = 1.31$, $p = 0.2715$; main effect of injury, $F_{1,70.1} = 5.16$, $p = 0.0261$, Cohen's $d = 0.2879$), but latencies were unaffected by injury during reversal training trials (injury by day interaction, $F_{3,155} = 0.25$, $p = 0.8582$; main effect of injury, $F_{1,71.4} = 2.48$, $p = 0.1199$) (Fig. 5A). Male and female mice had equivalent performance during standard training trials as assessed by the latency to find the platform (day by sex interaction, $F_{3,162} = 0.49$, $p = 0.6889$; main effect of sex, $F_{1,70.1} = 2.11$, $p = 0.1510$), but female mice had superior performance to male mice during reversal training trials, with reduced latency scores (day by sex interaction, $F_{3,155} = 1.42$, $p = 1.80$, $p = 0.1496$; main effect of sex, $F_{1,71.4} = 5.19$, $p = 0.0258$, Cohen's $d = 0.3427$) (Fig. 5B).

The distance mice swam before locating the platform was unaffected by injury during both standard (injury by day interaction, $F_{3,162} = 0.82$, $p = 0.4837$; main effect of injury, $F_{1,70} = 2.89$, $p = 0.0937$) and reversal (injury by day interaction, $F_{3,213} = 0.37$, $p = 0.7783$; main effect of injury, $F_{1,71} = 0.61$, $p = 0.4373$) training trials. Male and female mice swam the same distances before reaching the platform during both sets of training trials (Standard trials: sex by day interaction, $F_{3,162} = 0.99$, $p = 0.3993$, main effect of sex, $F_{1,70} = 1.02$, $p = 0.3154$; Reversal trials: sex by day interaction, $F_{3,213} = 2.57$, $p = 0.0553$, main effect of sex, $F_{1,71} = 3.92$, $p = 0.0517$) (data not shown).

There were no day by injury interaction effects on swim speed during the standard ($F_{3,163} = 0.66$, $p = 0.5779$) or reversal ($F_{3,153} = 0.11$, $p = 0.9566$) training trials, nor were there main effects of injury on swimming speed ($F_{1,68.7} = 0.16$, $p = 0.6918$; $F_{1,72.1} = 1.59$, $p = 0.2107$ for standard and reversal training trials, respectively) (data not shown). There was a main effect of sex on speed in the MWM during the standard training trials ($F_{1,68.7} = 4.03$, $p = 0.0485$, Cohen's $d = 0.3390$), with female mice swimming faster than male mice (Fig. 5C). Male and female mice had equivalent swim speeds during reversal training trials ($F_{1,72.1} = 0.66$, $p = 0.4208$).

The latency to find the visible platform on day 31 following injury was unaffected by injury status ($F_{1,71} = 1.07$, $p = 0.3052$) (Fig. 4A) or sex ($F_{1,71} = 2.33$, $p = 0.1316$) (Fig. 5B), and there was no interaction between the two factors ($F_{1,71} = 0.74$, $p = 0.3919$) (data not shown).

There was a significant sex by injury interaction effect on the time spent in the NW (correct) quadrant during the standard probe trial on day 21 following injury ($F_{1,71} = 4.38$, $p = 0.0400$). Bonferroni-corrected *post-hoc* tests showed that there were no sex differences in performance for the sham controls ($p = 1.000$), but the brain-injured female mice outperformed the brain-injured male mice in this test ($p = 0.0322$, Cohen's $d = 1.0870$), and also performed at a level equivalent to that observed in the sham female mice ($p = 1.000$; Fig. 6A). There was no sex by injury interaction effect on the time spent in the SE quadrant during the reversal probe trial on day 28 following injury ($F_{1,71} = 1.303$, $p = 0.2575$) nor were there main effects of injury ($F_{1,71} = 3.08$, $p = 0.0836$) or sex ($F_{1,71} = 1.59$, $p = 0.2108$) on this measure (Fig. 6B).

4. Discussion

The purpose of this study was to determine whether male and female mice differ on cognitive assessments of learning and memory following rCBI. Using a mouse model of repetitive brain injury, mice with five concussive injuries (24-h inter-injury interval) exhibited cognitive deficits lasting up to a month. Interestingly, both sexes exhibited equivalent impairment during the training trials of MWM testing, but female mice overall had superior spatial memory in comparison to male mice during the reversal trials of MWM testing. Moreover, injured female mice showed superior performance to the injured male mice on the standard post-training probe trials (Fig. 6A). Histochemical analysis by H & E staining showed no gross brain pathologies in either sex over four weeks following the injuries evaluated, but female mice had reduced GFAP immunoreactivity in the optic tract compared to their injured male counterparts. Astrocyte response to milder forms of injury is heterogeneous and more work is needed to define how CBI affects regional changes [23].

Injured mice had a longer righting reflex time (suggested to be a measure of loss of consciousness) than sham controls, particularly on the first day, but there was the curious observation that on subsequent days the righting reflex time of injured mice decreased. Other studies employing repeat concussions as a brain injury model have also observed a decrease in righting reflex time with repeated injuries [24,25] (but see [10,26]). There has been little speculation on the mechanism underlying this phenomenon, but Briggs and colleagues suggested that it may reflect an adaptation of the central nervous system to repeated head impacts [25].

Cognitive impairment, however, is common among athletes experiencing concussive brain injury. In accordance with previous studies [12–16], we observed that mice subjected to rCBI have deficits in learning and memory. On the MWM test of spatial memory, these deficits were significant during standard training (days 17–20). While we observed cognitive deficits in the MWM task, we were unable to find any differences between the performance of injured and sham-treated mice on the y-maze test of spontaneous alternation. Overall, these nonsignificant findings exemplify the difficulty of certain rodent tests to replicate human rCBI in an animal model. Indeed, Luo and colleagues [27] also reported no difference in spontaneous alternation after mild repetitive brain injury (CBI). On the other hand, studies involving mice with a single, severe TBI (CCI) found memory differences on the y-maze task [20].

Injured mice in this study showed pathology in the corpus callosum and the optic tract, confirming prior studies reporting neuropathology in these structures following multiple concussive injuries [4–6,12,13,24,27–29]. The central location of reactive astrocytes in the commissural region of the corpus callosum (Fig. 3B, CBI cases) is noted in several previous reports and is perhaps related to the CBI targeted to the midlateral region of the parietal cortex [30] and the relative higher density of GFAP-positive profiles in this region [31]. Microgliosis in the optic nerve of injured mice has also been reported following repetitive brain injury [5,24,28,29]. Xu and colleagues and Tzekov, et al. reported axonal degeneration in the optic nerves and optic tract and a loss of retinal ganglion cells following repetitive brain injury [5,28]. Because the y-maze and MWM both use visual cues to assist the animals, it is possible that performance on these tests may be affected by damage to the visual system. To eliminate a potential confound of visual impairment, we conducted visible platform trials at the end of MWM training. During the visible trials, performance of rCBI-mice was similar to that of the sham-treated mice. Based on these results, we can conclude that our results from behavioral testing were not due to visual system damage.

To date, no study has examined possible sex differences in mice on cognitive performance following repetitive concussive injury. Here, we report a main sex by injury interaction effect: female-injured mice outperformed male-injured mice on standard probe testing (Fig. 6A). Superior cognitive performance of female rodents compared to males following TBI was also reported by O'Connor and colleagues, who found that female rats performed slightly better on the Barnes maze of spatial memory than male rats during the one week immediately following weight-drop injury [32]. Most post-injury comparisons of male and female behavior have employed the CCI model and have concluded that the sexes have equivalent cognitive deficits following brain injury [20,33–35].

The current finding of better memory performance in injured female mice compared to male mice corroborates literature suggesting a role of female sex hormones in neuroprotection. In particular, previous studies have looked at the effect of female hormones on functional outcomes following brain injury [36]. Similar to our methods, Jones and colleagues [36] used the MWM to assess cognitive functioning in progesterone-treated, male mice following TBI. Overall, injured mice treated with progesterone displayed improved spatial memory, having a comparable performance to sham-treated mice on the MWM task. The MWM and y-maze are both tests of hippocampal-dependent learning and memory as injury to the hippocampus has been related to poor performance on these cognitive assessments. Progesterone has been demonstrated to have a regulatory effect on neurogenesis and cell death in the hippocampus [37]. Therefore, improved hippocampal-dependent memory, observed in the female-injured group during standard probe trials, may be evidence of female hormonal benefit.

In addition, we observed female-injured mice to have reduced GFAP immunoreactivity in the optic tract and shorter righting reflexes than male-injured mice on days 1, 2, 3, and 5 of CBI (statistical significance was reached on day 2). Based on the aforementioned studies, the observation of reduced neuropathology and the shortened righting reflexes seen in the female-injured group may also be attributed to the restorative effects of estrogen and progesterone. Indeed, a review by Chakrabarti and colleagues details a number of neuroprotective benefits of estrogen, including reduction of oxidative stress, increased angiogenesis, and suppression of cell death [38]. Moreover, studies have demonstrated the influence of estrogen and progesterone on proinflammatory cytokines after TBI. Specifically, varying doses of estrogen and progesterone decrease the levels of IL-1β, IL-6, and TNF-α, all of which contribute to brain edema and neuronal loss [39,40]. However, whether or not additional factors have a role has not been ruled out. Developmental, chromosomal, epigenetic and neurochemical and anatomical

sex differences may underlie bioeffects and behavioral differences [41–43].

Other studies, for example, have refuted the role of female sex hormones in neuroprotection. For example, Bruce-Keller and colleagues [44] did not observe any pathological differences in the areas of the cortex or hippocampus between estrogen-supplemented mice and vehicle (non-treated) mice following CCI. While the expression of IL-6 was significantly decreased by estrogen supplementation, a finding previously observed by Sarkaki and Soltani [39,40], a similar reduction was also noticed in the vehicle mice. Based on these results it could be suggested that estrogen does not carry a heavy role in neuroprotection. In addition to estrogen, multiple administrations of progesterone did not significantly alter the amount of cortical tissue sparing and edema in the rat brain [45]. Also, as levels of estrogen and progesterone vary with the estrus cycle, the estrus stage at the time of injury could have an impact on function and recovery. However, post-TBI deficits on motor tests and the MWM have been shown to be independent of estrus cycle stage at the time of injury in rats after CCI [34,35,46]. Overall, the question of whether female sex hormones provide the sole neuroprotective benefits requires further investigation, and other factors are almost certainly contributory.

In the current study, female mice displayed greater levels of activity than male mice. Previous research has suggested that the novelty of an environment may be linked to hyperactivity seen in female mice [20,47]. Tucker and colleagues [20] noticed that female mice were more active during their initial exposure to the open field test and the y-maze. Consistent with this earlier study, female mice traveled greater distances than male mice during their first exposure to the y-maze. Moreover, female mice recorded faster swimming speeds than males during the first week of the MWM but not during the following week of reversal training. However, factors other than novelty have been shown to induce hyperactivity in female mice. Hormonal studies have reported increased locomotor activity in mice treated with estrogen [48,49]. Experimental variables such as animal strain, type of behavioral test, and environmental lighting have also been documented to affect activity levels in rodents [50,51]. In all, it is critical for researchers to thoroughly examine their methods for all possible confounding variables before interpreting behavioral results.

The results presented in this study suggest that females fare better than males following concussive injury. However, studies investigating the effects of concussions in human athletes show mixed results. In a review of 51 articles pertaining to "gender and sports concussions," the majority of studies find that the incidence and outcomes of concussions are worse in female athletes [52]. Indeed, when examining sports played by both sexes, female athletes were found to have a greater concussion risk than male athletes [53,54]. Furthermore, the duration for female athletes to become asymptomatic, or without symptom, following concussion is longer than it is for males [55]. Still, other studies have shown the effects of concussions to be more detrimental in male athletes. In particular, a study by Covassin and colleagues [56] found that female athletes with a history of multiple concussions performed better than males with a similar concussion history on several neurocognitive variables such as processing speed, reaction time, and visual and verbal memory. Future studies will be needed to clarify the differences between the responses of males and females to concussions. One major limitation of sports-related concussion studies is their reliance on self-reported data. By allowing athletes to describe their own injuries, they may downplay the severity of their symptoms in order to appear healthy enough for gameplay. Ultimately, the understated responses from athletes can lead to mixed results, such as those from the previously described studies.

Funding

This work was supported by The Center for Neuroscience and Regenerative Medicine, 60855-300600-7.01.

Acknowledgments

The opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army, Department of Defense, the U.S. Government or the Uniformed Services University of the Health Sciences. The use of trade names does not constitute an official endorsement or approval of the use of such reagents or commercial hardware or software. This document may not be cited for purposes of advertisement.

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